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Review

Towards a method for cryopreservation of mosquito vectors of human pathogens

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A B S T R A C T

Mosquito-borne diseases are responsible for millions of human deaths every year, posing a massive burden on global public health. Mosquitoes transmit a variety of bacteria, parasites and viruses. Mosquito control efforts such as insecticide spraying can reduce mosquito populations, but they must be sustained in order to have long term impacts, can result in the evolution of insecticide resistance, are costly, and can have adverse human and environmental effects. Technological advances have allowed genetic manipulation of mosquitoes, including generation of those that are still susceptible to insecticides, which has greatly increased the number of mosquito strains and lines available to the scientific research community. This generates an associated challenge, because rearing and maintaining unique mosquito lines requires time, money and facilities, and long-term maintenance can lead to adaptation to specific laboratory conditions, resulting in mosquito lines that are distinct from their wild-type counterparts. Additionally, continuous rearing of transgenic lines can lead to loss of genetic markers, genes and/or phenotypes. Cryopreservation of valuable mosquito lines could help circumvent these limitations and allow researchers to reduce the cost of rearing multiple lines simultaneously, maintain low passage number transgenic mosquitoes, and bank lines not currently being used. Additionally, mosquito cryopreservation could allow researchers to access the same mosquito lines, limiting the impact of unique laboratory or field conditions. Successful cryopreservation of mosquitoes would expand the field of mosquito research and could ultimately lead to advances that would reduce the burden of mosquito-borne diseases, possibly through rear-and-release strategies to overcome mosquito insecticide resistance. Cryopreservation techniques have been developed for some insect groups, including but not limited to fruit flies, silkworms and other moth species, and honeybees. Recent advances within the cryopreservation field, along with success with other insects suggest that cryopreservation of mosquitoes may be a feasible method for preserving valuable scientific and public health resources. In this review, we will provide an overview of basic mosquito biology, the current state of and advances within insect cryopreservation, and a proposed approach toward cryopreservation of *Anopheles stephensi* mosquitoes.

1. Mosquitoes

While mosquitoes are similar to other insects that have been successfully cryopreserved, they also have their own unique biological characteristics. These include their aquatic and terrestrial lifecycle, and their diapause, quiescence, cold tolerance, and desiccation tolerance mechanisms. Understanding these mechanisms that mosquitoes utilize is important to successfully cryopreserve and recover mosquitoes.

1.1. Taxonomy

Mosquitoes are members of the order Diptera which contains two-winged flies. Within Diptera, mosquitoes make up the Culicidae family, present throughout most of the world, but most abundant in tropical and temperate regions. The Culicidae family is divided into two subfamilies, Anophelinae and Culicinae [162,178]. There are 112 genera of mosquitoes. These include *Aedes*, *Anopheles*, and *Culex*. Culicidae is monophyletic, however deeper relationships within the family are poorly resolved due to lack of taxonomic sampling [162].

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1.2. Basic life cycle

Mosquitoes have four distinct life stages: egg, larva, pupa and adult (Fig. 1) [224]. For most mosquitoes, females lay their eggs in water, although the particular type of aquatic environment varies greatly by species [47]. The morphology of the eggs themselves, and the way they are laid, vary by species as well. *Culex* eggs are laid in rafts on the surface of water that tends to be relatively high in organic content, *Mansonia* eggs are often attached to the undersurface of lily pads [138], and *Aedes* lay their eggs on damp surfaces or near the water's edge [47]. Mosquito eggs hatch into larvae which live in water and breathe air at the surface. Larvae go through four stages (instars; see Fig. 1, "L1-L4") that increase in size while consuming organic matter and microorganisms in the water where they live [124,179,225]. The instars can be delineated based on size, number and location of seta (hairlike structures), development of clypeal hair, coloration patterns, and other morphological variables depending on species [61,77,167].

After the fourth instar, the larva pupate [21,106,224,225]. The resulting pupae are motile, but non-feeding. During this stage, mosquitoes undergo complete metamorphosis and emerge as adults, which rest on the surface of the water to dry and harden, before leaving the water entirely. Adult female mosquitoes forage for sugar [52,182], blood-feed and mate a few days after emerging, although this varies greatly by species [175,186,219]. Propagating and maintaining mosquitoes in the laboratory is complex and time-consuming, and its success is dependent on many variables including temperature, humidity, light, diet, food density, etc. [22,73,89]. From a basic understanding of the mosquito life cycle and the known cryobiology of mosquitoes, it seems most likely that early larval stages are the most attractive targets for efforts toward cryopreservation due to their small size and aquatic nature [119].

1.3. Mosquitoes as disease vectors

Mosquitoes transmit a variety of bacteria, viruses and parasites, resulting in over a million deaths each year [45]. Malaria is caused by the *Plasmodium* parasite which is transmitted by female *Anopheles* mosquitoes. Half the world's population is at risk for malaria. Over 200 million infections and almost half a million deaths annually are attributable to malaria, making it the deadliest mosquito-borne disease [2]. Viruses such as dengue, yellow fever, chikungunya and Zika are mainly transmitted by *Aedes* mosquitoes in Africa, Southeast Asia and Latin America. Other viruses, such as West Nile, Saint Louis encephalitis, and Western equine encephalitis are transmitted mainly by *Culex* mosquitoes. All of these viruses infect humans, leading to millions of infections, and a massive burden on public health systems [3,28]. For a mosquito to transmit these pathogens, they usually must feed on an infected individual (horizontal transmission), however there are also instances of vertical transmission (female to egg) [7]. Vertical transmission allows pathogens to persist within mosquitoes without an intermediate host. Mosquitoes in diapause can also serve as a reservoir, allowing pathogens to re-emerge the next season when conditions are favorable again [51]. Importantly, mosquitoes can play critical roles in nature [63]. Certain mosquito species are part of the food chain to some dragonfly, bat, fish and bird species [17,18,72,176], and others are pollinators of orchids and tansies [109,149].

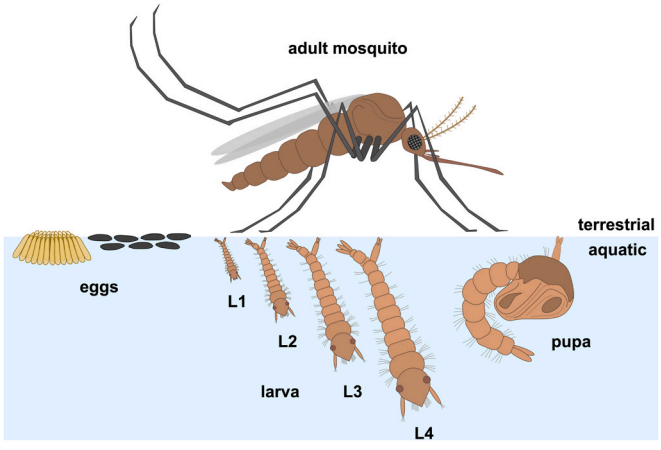

Fig. 1. Mosquito life stages. Life cycle of the mosquito from eggs, through the four larva instar stages, to pupa, then adult mosquito.

1.4. Genetically altered mosquitoes

For over 20 years, technological advances have allowed researchers to genetically modify mosquitoes [158]. This includes alterations to reduce/suppress wild mosquito populations, altering male mosquitoes to carry repressible lethal genes causing their offspring to die [100,217], altering immune genes making mosquitoes resistant to infection by a wide array of viruses [67,218], altering genes to investigate insecticide resistance [6], host odor sensing [50], or otherwise study aspects of mosquito biology [79,218]. While these techniques are promising, especially combined with more genetically stable manipulations (such as CRISPR) and gene-drive strategies [158], continued rearing of these mosquito populations often leads to a repression or loss of the desired genetic modification or phenotype [67,154]. Additionally, mosquitoes are being used to produce a malaria vaccine, which requires continuous maintenance of the colony, making it susceptible to genetic drift and adaptation [84]. The ability to cryopreserve these genetically altered or unique mosquito strains would allow them to be preserved indefinitely and revived before genetic markers or phenotypes are lost. Additionally, cryopreservation would aid in the delivery of getting mosquitoes to where they are needed, as shipping preserved samples is easier than live biological material [74,180].

1.5. Diapause, quiescence, and tolerance mechanisms

Mosquitoes have developed many mechanisms to survive unfavorable environmental conditions (often too cold or too dry) [51,54]. Mosquito diapause is dormancy that is hormonally programmed before its onset and is not immediately terminated when conditions become favorable. Diapause can occur at each mosquito life stage (egg, larva, pupa, adult), however most species only diapause in one of the stages (e.g. *Aedes albopictus* as egg, *Anopheles gambiae* as adults) [51]. For example, short day lengths cause female *Ae. albopictus* to lay eggs that will fail to hatch, even if submerged in water, because the diapause was programmed environmentally during the mother's life, and not due to dry egg conditions [14]. Eggs are only able to respond to the stimulus of water after a set amount of time has passed. Unlike diapause, which is an advanced programming, quiescence is a dormancy that is elicited in direct response to unfavorable conditions and ends when conditions become favorable again [145]. For example, *Aedes aegypti* eggs that are laid above the water line are quiescent, because as soon as they are immersed in water, development will immediately begin [153]. Additionally, *Ae. aegypti* lack diapause, which restricts them to favorable habitats (tropical and subtropical).

As part of diapause and quiescence, mosquitoes have developed tolerance mechanisms [94,98,163,199]. Many mosquito species have desiccation tolerance mechanisms that will prevent their embryos/eggs from drying out during unfavorable conditions (diapausing eggs are often more desiccation resistant than non-diapausing eggs) [64,98,199,203]. Desiccation resistance is enhanced in some species by increasing hydrocarbons on the surface of eggs [64,203], or the cuticular surface of adult [23]. Mosquitoes also have cold tolerance mechanisms to survive cold conditions [94,163,202]. Diapausing adult *Cx. pipiens* have increased trehalose levels [23], and *Ae. aegypti* larvae and adults have mechanisms to prevent failure of ion transport processes at low

temperatures [94]. It is important to understand these various mechanisms, in order to more successfully cryopreserve and recover mosquitoes.

2. Cryobiological basics relevant to insect cryopreservation

The goal of freezing any cell, tissue, or organism is to put it in a state of suspended animation. The sample is brought to a low enough temperature that metabolism is minimized such that cells will not require resources or produce excessive waste products over a desired period of storage, with the ultimate goal of bringing the sample back to a temperature at which it can continue to live [19,220]. The ability to do this successfully relies on a variety of cryobiological principles that must be considered on a cell, tissue, or species-specific basis (if freezing whole organisms) [75,151].

In order to avoid damage during freezing [150], one must prevent ice crystal formation within the cells, which is achieved by dehydration of cells through the addition of cryoprotective agents to the freezing medium [59]. Non-penetrating cryoprotectants elicit this effect by creating a concentration gradient that draws water out of the cells [222]. Penetrating cryoprotects, which are typically in higher concentrations than non-penetrating cryoprotectants, initially contribute to this concentration gradient but this will diminish as they move into the cell where they replace some of the water that is being lost [25,59]. The presence of the penetrating cryoprotectant within the cell decreases the chances of ice crystal formation during the cooling process. At high enough concentrations and after long periods of time, nearly all cryoprotectants can be toxic to cells [25]. Thus, choosing the optimal cryoprotectant for a biological system is based on its ability to cross the plasma membrane, the rate at which it crosses the membrane, and how long the cells can be exposed to it at the selected concentration before causing lethality [25,59]. This is largely dictated by the size of the molecule, though it is not the only factor.

Cooling rate is also critical to the survival of cells [88,104,110]. As a suspension is cooled and ice forms, the remaining solution becomes increasingly concentrated which can elicit toxic effects on the cells, tissues, or organism being frozen [25]. The ideal cooling rate for a given biological unit is one that balances the time it takes to gently dehydrate the cells, against leaving the sample in a highly concentrated solution for a period of time that is lethal [88]. An effective cooling rate also decreases the likelihood of intracellular ice formation while preventing rapid changes in cell volume that may be damaging to the cells or organism. Many freezing protocols include an equilibration period prior to cooling to allow for some dehydration and penetration of cryoprotectant to avoid injury once cooling begins [66,69]. In slow cooling, the exit of water, and entry of the cryoprotectant (at low 1–2 M concentration) are in equilibrium. Protocols for successfully slow freezing a wide variety of cells and tissues have been reported.

An approach that aims to avoid ice crystal formation all together is vitrification [62,88,170]. Often referred to as ultra-rapid cooling, vitrification is achieved with high concentration of cryoprotectants (~6–8 M), by cooling suspensions so quickly (10,000°+/min) that rather than ice crystal formation, solutions become so glassy and viscous that they function as a solid. Vitrification still requires an equilibration period prior to rapid cooling and is often done with much higher concentrations of cryoprotectant, though that may not be necessary [170]. This approach requires a rapid warming of the solution to prevent ice crystal formation as suspensions go from a vitrified to a fluid state. A variation of vitrification is performed with a short incubation in a lower concentration cryoprotectant (~2 M), followed by a high concentration (~8 M) incubation, which further removes intracellular water [129,188]. In the second technique, the organism must be able to survive the long exposure times required by this protocol [161]. Rapid warming will be discussed later in this review.

Much of the foundational cryobiological research and founding principles were described from research that was conducted on single cell types [151,198,214]. Cryopreservation of whole, multi-cellular organisms, like mosquitoes, provide an additional layer of complexity, though basic cryobiological principles still apply. Indeed, this has been achieved before with other insect species [223], and there are a handful of other animals that are completely frozen in their natural environments (e.g. wood frogs) [43]. Here we will consider those successes and the challenges specific to mosquitoes relative to the basic cryobiological principles we have discussed.

2.1. Overview of species studied

The vast majority of studies on cryopreservation of insects has focused on silkworms and other moth species, fruit flies and honeybees, however studies on other species exist (house fly [112,165,210], ladybird beetles [82,156], spined soldier bugs [58], fireflies [117], etc.). Silkworms are model organisms for arthropod biology, due to their small size and ease of culture [134]. Their genome is fully sequenced, and has recently been genetically modified to produce proteins, drugs, and spider silk [134]. Fruit flies are model organisms with fully sequenced genomes, techniques to modify and manipulate their genomes, biological and molecular tools (cell lines, gene arrays, antibodies, etc.) and decades of research making them an ideal system to study cryopreservation [107,223]. Honeybees are important pollinators and critical to agriculture crop production [49]. Unfortunately, they are suffering massive population declines due to habitat loss, pesticides, parasites, pathogens, and increasing winter die-off rates [49,116]. The ability to cryopreserve and create a biorepository of genetically diverse honeybee strains would allow breeding and reintroduction, potentially reversing their decline in populations and endangered status [143]. While mosquitoes are genetically and biologically distinct from each of these species, building off the work developed to cryopreserve these species, we hope to inform a protocol to successfully cryopreserve mosquitoes. Likely the most relevant and important work to reference will be that using the same developmental stage that we choose for mosquitoes (larva, egg, embryo, etc.).

2.2. Selecting a suitable cryo-tolerant developmental stage

A critical variable for insect cryopreservation is selecting a suitable developmental stage (ovary, embryo, egg, semen, larva, etc.) [90,115]. Depending on the species, certain stages may be more compatible with cryopreservation than others. Within the developmental stages, there are subtle changes that can also greatly impact cryopreservation success. For example, Mexican fruit fly embryos at the mouth hook formation stage contain the ideal balance between yolk depletion and cuticle development, are more tolerant to cryopreservation than both earlier and later embryo stages [159]. One of the key elements is understanding the permeability of the tissue(s) to cryoprotectants and water at a variety of developmental stages. Permeability and chilling sensitivity work in an interactive fashion where once a candidate developmental stage that is permeable is chosen, the chilling sensitivity of that stage and the toxicity of a variety of cryoprotectants at various concentrations must be determined empirically. This must be repeated for all developmental stages of interest as the complexity of tissues and structures varies greatly and will have an impact on survival.

Certain developmental stages are more compatible with cryopreservation than others. For example, for the New World screwworm the embryo is the best stage to cryopreserve [113]. However, it is critical to collect embryos just prior to the development of the embryonic exoskeleton [24]. When younger embryos are used, embryonic development is stunted after recovery [24]. Older embryos have already developed their embryonic exoskeleton, making them impermeable to cryoprotective agents [115]. Additionally, survivability of cryopreserved greater wax moth embryos is highly dependent on the stage (and therefore eggshell permeability) of embryo used [4]. These two examples highlight the crucial role that proper developmental stage and

timing plays on the ability to successfully cryopreserve and recover insect species.

Cryopreservation of silkworms has focused primarily on freezing ovaries, eggs, or sperm [16,96,195]. Honeybee cryopreservation efforts focus primarily on freezing drone sperm [9,212,213], however there has been some work to evaluate chilling sensitivity of honeybee embryos, and embryo preparation prior to cryopreservation [41,76]. Importantly, freezing ovaries, eggs or sperm, requires artificial insemination or *in vitro* embryo production after thawing and recovery. For some species (including silkworms and honeybees), artificial insemination is a well-established and successful procedure [39,195], however, while this technique has been demonstrated for mosquitoes [34,83,216], it has not been robustly validated nor is it in wide use. Unlike silkworms and honeybees, the majority of fruit fly cryopreservation focuses on the larval stage [107,108,189]. Like mosquitoes, fruit flies lay fertilized eggs, which progress through multiple larval instars, before becoming a pupa, then an adult. Unlike mosquitoes, the fruit fly larval and pupa stages do not occur in water.

2.3. Membrane permeabilization

Many insect embryos and eggs have barriers specifically to prevent the loss of water [115], making penetration of cryoprotectant and dehydration in preparation for freezing problematic. These barriers can include a chorion, a wax layer, a vitelline membrane (the inner layer of the chorion), or other proteinaceous or lipid layers which can vary in their hydrocarbon composition [12,13,64,91,127,133]. Membranes and chorions can be removed/permeabilized with methanol or isopropanol, alkane solvents (such as hexane or heptane), or bleach solutions [64,206,207]. The concentrations, time of treatment, and other variables are optimized for each species and developmental stage. By far, it is preferable to choose a developmental stage that is permeable to water and cryoprotectants, if at all possible.

2.4. Cryoprotective agents

Penetrating and non-penetrating cryoprotectants are often used in combination for insect cryopreservation [59,215]. Common penetrating cryoprotectants are usually small molecular weight molecules that can solubilize through the membrane, such as ethylene glycol, glycerol, and dimethylsulfoxide (DMSO) [93]. In contrast, larger molecular weight molecules, such as various sugars (sucrose, trehalose [60,86,144,190], etc.) act as non-penetrating cryoprotectants. The majority of insect cryopreservation has been done using ethylene glycol, often with the addition of polyethylene glycol or trehalose to reduce fracturing of the vitrified solution during rapid cooling [120,122,210].

2.5. Cooling rates

As mentioned above, cooling can be achieved slowly using osmotic equilibrium, or rapidly, which results in vitrification [62,170], but both techniques attempt to prevent the critically damaging formation of intracellular ice [88,130]. Vitrification can be achieved by exposing organisms to a high concentration of the cryoprotective agent in one step, or in a two-step variation (used for the New World screwworm and *Drosophila*), by first incubating the organism in a lower concentration of cryoprotectant to allow time for the cryoprotectant to enter the cell, then further dehydrating it by exposure to a higher concentration (for a shorter period of time) to remove additional intracellular water, thereby increasing the intracellular concentration of the cryoprotectant [115,129].

2.6. Storage, thawing and recovery from storage

After cryoprotectant loading, organisms are rapidly cooled, then placed in liquid nitrogen for storage [110,155,180,201], however there are new studies attempting to develop dry storage of lyophilized material [121,135,171]. Thawing and recovery from storage is as important (possibly more important!) as cooling and must occur rapidly to prevent intracellular ice formation [78,104]. Additionally, the cryoprotectant must be removed and replaced with a biologically appropriate solution. This is often achieved by placing the organism in a cell culture medium containing a non-penetrating cryoprotectant (such as trehalose or sucrose [27]) which prevents over rehydration of cells by a rapid influx of water while the cryoprotectant, which moves across membranes more slowly than water, is being unloaded from the cells. Thawing and/or warming procedures must be optimized to prevent lethal changes of cell volumes. One way to combat over rehydration, and potential rupture of cells, is a stepwise decrease in the concentration of the non-penetrating cryoprotectant [25,59].

2.7. Viability and phenotypic assessments

Successful insect cryopreservation includes evaluating and ensuring the cryopreserved insects are viable, and similar (biologically, morphologically, behaviorally, etc.) to their control non-cryopreserved counterparts. To assess viability of cryopreserved honeybee sperm cells, groups have used live-dead stains [40,152] and TUNEL assays to evaluate genomic integrity [213]. Many different metrics and tests have been used to evaluate post-thaw viable insect samples, including reproductive tests (fertility, fecundity, hatching and mating propensity, number of eggs per clutch, etc.) [68,114,172], phenotypic tests (size, weight, protein content, metabolic rates, etc.) [108,213], physical tests (motility, flight ability and duration) [9,10,114], along with newer molecular genetic effects (gene expression, epigenetics, etc.) [37,108,160]. In some insect species cryopreservation impacts an aspect of the organism's biology (e.g. low pupal weight), but that is not seen in the progeny of the next generation, indicating that some consequences of cryopreservation are transient and do not alter the organism long-term [113]. These types of assessments will be critical to validation of any protocol that is developed particularly for biobanking mosquitoes where consistency across time/generations and avoidance of adaptation [166], gene flow and genetic drift are the goal [48,71,123].

2.8. New technologies

The field of cryobiology has advanced greatly within the last ten years [36,103,209]. Many of these new and improved technologies make cryopreservation of certain organisms and species now possible. One of these advances is infrared laser warming, which can warm samples by up to ~10^7 °C/min (standard rates depending on the method can be 100 to 10,000 °C/min) [147]. This ultra-rapid warming greatly decreases the chance of devitrification (ice formation) during recovery from storage [46,95,103]. As mentioned, cryoinjury often occurs during the warming process [69], and laser warming provides a controlled way to increase the temperature more rapidly than could be done through convective warming on the bench. There have also been advances in nanowarming, which is both uniform and rapid [70,125,183]. These technologies coupled with newer devices for vitrification in minimum volumes of media (as little as 1 μL; Cryolock®, Kitazato, Japan) [36,187], which minimizes the chances of ice crystal formation during the vitrification or warming process, sets the stage for new successes, particularly for organisms like mosquitoes for which little research has been published in the past decade. There have also been advances in the development and use of macromolecular cryoprotectants [139,140,191,192], antifreeze proteins [42,105,141,164,200] and ice recrystallization inhibitors [142], which helps ensure intracellular ice does not form by surrounding ice nuclei and preventing them from growing during thawing [35]. Importantly, these newer techniques and devices are not always widely accessible in the cryobiology field, however they can be utilized as proof-of-concept with new or difficult to cryopreserve species and organisms.

2.9. Biobanks

It is critical that cryopreservation techniques developed for mosquitoes are easily accessible and implementable by existing and new biobanks. Biodefense and Emerging Infections Research Resources Repository (BEI Resources) and American Type Culture Collection (ATCC) are some of the largest repositories for scientific reagents [1,137]. There are additional biorepositories such as the National Animal Germplasm Program [29,30], and the National Ecological Observatory Network (NEON) Biorepository [111], which is expanding to accommodate cryopreserved collections, and those that specialize in specific insects and insect cells, such as The Tick Cell Biobank [20], the Bloomington Drosophila Stock Center at Indiana University [132], and the Agricultural Research Service Midwestern Livestock Insects Research Unit which has distributed cryopreserved screwworm fly eggs to additional repository centers [194]. Historically, researchers share mosquito lines directly with one another; or when not available, from the Malaria Research and Reference Reagent Resource Center (MR4) through BEI Resources [5,221], or capture them from the field and establish new colonies. Development of cryopreservation techniques for mosquitoes would allow for standardized banking, recovery and rearing of mosquito lines across laboratories, a critical component of mosquito research.

3. Proposed approach for cryopreservation of *An. stephensi*

Here we propose a protocol for the cryopreservation of *Anopheles stephensi*, a common laboratory strain and important malaria vector, as a case study for mosquito cryopreservation protocol development.

3.1. Challenges to mosquito cryopreservation

As discussed above, there is a critical need to develop cryopreservation techniques for mosquitoes. However, unlike the model organisms discussed above, there are distinct challenges involved with cryopreserving mosquitoes [119,120,131,174,206,207]. Unlike the other organisms, the early stages of mosquito development (egg, larva and pupa) are all partially or entirely aquatic. Because of their complete dependence on water, the ability to dehydrate and prepare them for cryopreservation is more sensitive than fruit fly or worm larva, which are not aquatic. However, aquatic larva of other species including shrimp [11,55,57], ragworm [146,211], oysters [193,204], and more have been successfully cryopreserved [56].

To undergo cryopreservation all organisms must tolerate dehydration to some extent, but the cryopreservation process can be modified to take into account the biophysical needs of the cells and tissues. For example, many freshwater aquatic organisms have an internal osmolality of around 300 mOsm. Therefore, they must defend their growing offspring from their low osmotic surroundings (~40 mOsm) by employing impermeable membranes or waxy coatings. An excellent example of this was the zebrafish embryo whose membranes were impermeable to most cryoprotectants and had a very low water permeability, as well [80,81]. Overcoming these barriers, necessitated microinjecting cryoprotectants into the yolk and using laser warming to reduce ice crystal damage of cryopreserved samples [102]. As long as these restrictions in water and cryoprotectant permeability were understood, they could be overcome, and the organisms successfully cryopreserved. An important consideration for organisms that survive in moist air, such as the fruit fly–once their permeability barriers are breached, they must be cultured under isosmotic conditions to achieve further development [129].

The ability of aquatic larvae to regulate their osmotic pressure (balance of water and salts), is critical to development, metabolism, and survival. Larval osmoregulation has been studied in land-crabs [99], multiple fish species [173,208], shrimp [32,33,92], and both *Aedes* and *Anopheles* mosquito species [168,181,205]. The midgut, Malpighian tubules, gastric caecum, rectum and anal papillae have all been identified as osmoregulatory organs in mosquito larvae [8,44,97,128,136,148], and therefore it is critical that cryopreservation does not alter their osmoregulatory capabilities. As a new model system is explored for cryopreservation, solid biophysical parameters must be determined to understand their sensitivity to chilling, water and cryoprotectant permeabilities, the osmotically active volume and whether the cells have water channels. With these parameters in hand, many decisions can be quickly determined as to the optimal pathway for cryopreservation and biophysical models can be created to help guide the process.

3.2. Mosquito life cycle stage

Like other insects, mosquito eggs have barriers to prevent water loss [65,203], making them impermeable to standard cryoprotective agents such as ethylene glycol [120,174,206,207]. Mosquito eggs have a wax layer on the surface of their vitelline membrane, and hours after being laid, they develop a second permeability barrier which is refractory to removal by hexanes [131]. Additionally, there have been no effective methods developed to remove the second barrier without killing the eggs [118], making cryopreservation of mosquito eggs essentially impossible [120]. For these reasons, the limited research on mosquito cryopreservation has focused instead on the larval stage. First instar mosquito larva are permeable to rhodamine B, suggesting they would also be permeable to much lower molecular weight cryoprotectants (e.g. ethylene glycol and methanol), making them a more promising developmental stage to pursue [120].

3.3. Cryopreservation variables

While little information for cryopreservation of *Anopheles stephensi* is available, some information is available regarding *An. gambiae* [119,120], a closed related and highly similar species, meaning the results and techniques will likely be translatable directly to *An. stephensi.* For instance, it has been shown using proton nuclear magnetic resonance (NMR) that first instar *Anopheles gambiae* larvae are highly permeable to both ethylene glycol and methanol with limited toxicity without any permeabilization treatment required [120], making these ideal cryoprotective agents. Additionally, the kinetics and tolerance of *An. gambiae* larva to dehydration has quantitatively been measured in air gravimetrically [119]. It is found that at room temperature, they can survive the removal of >75% of their water, and survival rates increase with pre-treatment of trehalose or sucrose.

An. gambiae larvae have high chill sensitivity, which means in order to bypass chilling injury, they will require a rapid cooling/vitrification approach to be cryopreserved. Generally, vitrification requires high concentrations of cryoprotective agents within the cells, but with small volumes and laser warming, the concentrations can be as low as 3–3.5 M [46]. Pairing this with ice recrystallization inhibitors (discussed above) [35], such as small carbohydrate molecules which can prevent recrystallization at much lower concentrations than other compounds, might potentially allow use of even lower concentrations of penetrating cryoprotectants [35].

Although each experimental step will have to be examined individually, previous studies support pre-treating *An. stephensi* first instar larva with 0.5 M trehalose. Timing for this step can be modeled from previous studies by Mazur and colleagues [119]. This pre-treatment dehydration step may remove a majority of intracellular water. Often with laser-warming, dehydration alone is sufficient [46,95], however, superior results are predicted with modest concentrations of ethylene glycol and trehalose as step 2. Again, this step is modeled by Mazur's work, which found 1.5 M concentrations of ethylene glycol and methanol were sufficient to permeate *Anopheles* larva [120]. Additionally, with faster warming techniques, such as laser gold warming, and pre-dehydration steps, even lower concentrations of cryoprotective agents may be possible [103]. Once larvae are loaded with vitrification solutions, rapid freezing is proposed and achievable by loading ≤2 μl drops with 2 or 3

larvae onto a Cryolock® and plunged into liquid nitrogen [36,187]. Once vitrified, the Cryolock® will be transferred to canes and stored in liquid nitrogen until warming. Novel recovery techniques using laser warming or radio frequency warming [126] is suggested to recover the larvae from the vitrified state after which they will be slowly rehydrated, cultured and initially evaluated for viability by the detection of movement.

3.4. Evaluation of cryopreserved mosquitoes

Gross measurements of permeability of cryoprotectant to *Anopheles* eggs and larvae via whole larvae proton NMR, along with viability assays following rehydration [119,120] provide methods as starting points for cryopreservation evaluation. Assuming recovered larva are viable and motile, these measures, combined with fecundity, lifespan, and clutch counts are reasonable evaluation tools for method development [31,185,197]. The transfer of methods from a proof-of-concept stage to broad applicability is better supported by the addition of molecular assays, such as a microtiter plate assay for a protein or glycan, mass spectrometry assay for a polar lipid or protein, or NMR spectrum, since these can be performed in multiple academic and biorepository settings. Ideally, one or more assays would be deployed at each key step in the cryopreservation and recovery process; each would include acceptable ranges of data that predict success of protocol implementation.

Here, we propose an exploration of assays for each key step in this process; 1) suitability of harvested larvae for cryopreservation; 2) dehydration and cryoprotectant loading; 3) rehydration after storage, and 4) colony stability, including assessment of long-term stability of preserved materials (percent recovery over time) and number of survivors required to regenerate colony. Previous work has suggested no fewer than 100 to minimize the risk of inbreeding depression [157,166,169]. Dehydration and cryoprotectant loading were previously assayed using NMR to follow the loss of water and replacement with either ethylene glycol or methanol [120]. This is an easily deployed assay; we propose continuing this assay along with generation of NMR reference figures associated with batch-to-batch data sheets each time this step is deployed. Similarly, rehydration associated with survival is likely to correlate with NMR spectral analysis of the solute in which larvae are bathed in for rehydration [120]. Rehydration was associated with high survival when a mixture of PBS and water (1:1; 0.13Osm/L) was used for rehydration [119]. It is likely that NMR analysis of the rehydration media demonstrating increasing concentration of urea, creatinine, Na, Cl or K (components of mosquito urine) [26,38], and/or cryoprotectant will be associated with higher survival, and again can be readily obtained and used as reference data for this step.

Assays to support steps 1 and 4 pose more of a challenge. Total carbohydrate, protein, lipid or glycogen concentrations associated with larval weight may be predictive of acceptability of a larval harvest for cryopreservation and thus determining an acceptable range of gross protein content may be a good baseline measurement before proceeding [15,177,196]. Discreet assays of specific proteins, such as chitin concentration in the peritrophic matrix membrane, may be more suitable and should be additionally explored [53]. Similarly, metabolic markers may be the most robust [87]. Indeed, studies previously conducted by us demonstrated increased phosphatidyl choline (PC) and riboflavin and decreased hydroxy-eicosatetraenoic acid associated with reduced fitness in *An. gambiae* mosquitoes fed with food contaminated with *Mycobacterium ulcerans* [85]. Phospholipid biosynthesis and alterations in other lipid and amino acid pathways were the most altered pathways associated with genetically or small molecule inhibitor altered *An. stephensi* [184]. These polar lipid alterations correlated with changes in lifespan and/or fecundity [184]. The topology of *An. stephensi* establishes specific PC isomers in typical populations [101], and it is believed that the role of trehalose in improving successful dehydration of *Anopheles* mosquitoes is due to the intercalation of trehalose into the polar phospholipid membrane of mosquito larvae [119]. Cumulatively, this supports the exploration of PC and other metabolites to measure and exploit for the development of quality control assays for these remaining steps and the entire cryopreservation process.

4. Future directions

In closing, our protocol template for *An. stephensi* cryopreservation remains similar to that provided by Leopold and colleagues [115] and includes optimization steps and quality control checkpoint assays that will permit broad use of the fully developed protocol in many academic and biorepository environments. Based off the extensive cryopreservation research optimized with other insect species, the preliminary work performed on mosquitoes, and the advances within the cryobiology field, we are confident that successful cryopreservation and robust recovery of *An. stephensi* mosquitoes is possible. Successful pilot studies with *An. stephensi* mosquitoes may be used to build cryopreservation protocols for other mosquito species (*An. gambiae, Culex* spp., *Aedes* spp., etc.) and test the translatability and scalability of any established mosquito preservation techniques.

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